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THE CONNECTIVE TISSUES IN
MEDICINE AND SURGERY*

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CURRENT research activity in the connective tissue field owes much of its initial vigor to the enticing concept of the "connective tissue" or "collagen" diseases developed by Klinge, Klemperer and others. I wish it were possible to claim that we understand how connective tissue is involved in these conditions or at least to report significant progress in this direction. Witness the gap between infection with the B hemolytic streptococcus and the scarred distorted heart valves in rheumatic fever. In the opinion of this writer there is little evidence as yet that collagen is involved in any way, other than as an innocent bystander or in the healing accompanying injury. The significance of the variable and often very minimal changes observed in the ground substances are yet to be evaluated. Even in scleroderma, a disease manifesting a gross alteration in connective tissue, both qualitative and quantitative informa-

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tion concerning the changes in the tissue components is still lacking. There is only histological evidence, to date, for an increase in collagen content in the skin. Although fibrinoid has long been considered the hallmark of connective tissue diseases there is still no general agreement as to either its composition or its pathogenetic significance¹⁻⁴. Is it an important part of the disease process with regard to manifestations? Is it a good lead to the mechanism? Or does it represent burned-out ashes of a relatively non-specific process?

Perhaps one measure of the current state of our knowledge concerning pathology of the connective tissue is our understanding of the nature of that most legitimate of all connective tissue diseases, scurvy. The cause and cure are known; an ideal experimental animal, the guinea pig, is readily available; the disease may be turned on and off at will and its severity is measurable. Since the original observations of Wolbach in the early 1930's, progress with regard to pathogenesis has been painfully slow. We have more descriptive information of a biochemical nature and several interesting hypotheses but the mechanisms responsible for manifestations of scurvy, such as failure to heal, hemorrhage, loosening of teeth and muscle attachments, etc. are still obscure. The biochemical functions of vitamin C are still unknown. Our difficulties with the pathogenesis of the rheumatic diseases are therefore not surprising.

Whether the connective tissue is a prime reactor in the rheumatic diseases or involved secondarily, the crippling deformities and much of the functional impairment result from failure of the newly forming connective tissue to reproduce the normal structures. Although this healing phase may be non-specific it is none the less a major feature of the course and ultimate consequences of most of this group of diseases.

This paper will deal with two specific problems related to healing, in which the writer has been involved, namely, collagen formation and wound contraction. There are a number of excellent connective tissue reviews now available⁵⁻¹⁶.

FIBROGENESIS OF COLLAGEN

The mechanism of collagen formation has intrigued histologists, pathologists and surgeons for many decades. Much discussion has centered on the question as to whether or not the collagen fibrils are formed intra- or extracellularly. This old argument is still viable and difficult

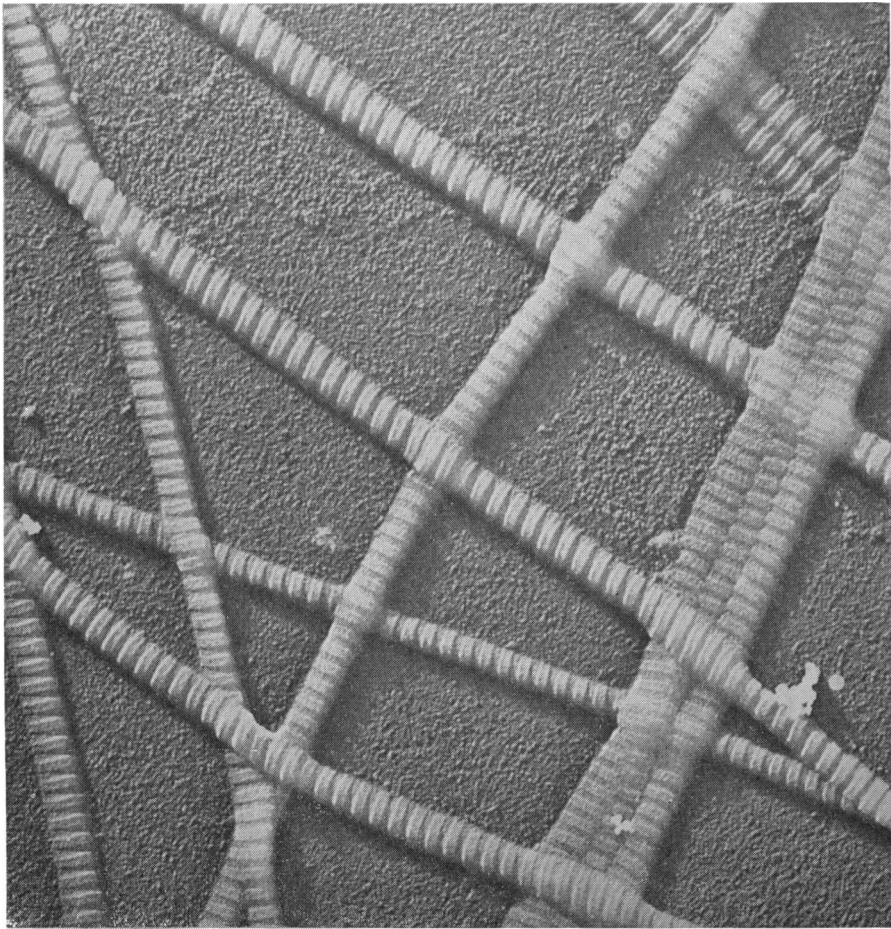


PLATE I—Electron micrograph of collagen fibrils from bovine skin. Shadowed with chromium. 47,000 X.

to settle conclusively—even by electron microscopy. The demonstrations of collagen fibrils within the fibroblasts, clearly observed on occasion in electron micrographs of thin sections^{13, 17a}, may be interpreted as representing collagen enveloped by the cell in its passage through the tightly packed “jungle” of fibrils. They may also represent precipitation of fibrils within a vacuole of extracellular matrix which had been pinocytosed by the cell. Light optical studies of fibroblasts in tissue culture have suggested fibrous structures coming directly from the cells; these have been widely interpreted as representing collagen fibers formed within the cell and in the process of being extruded^{17b}. Electron

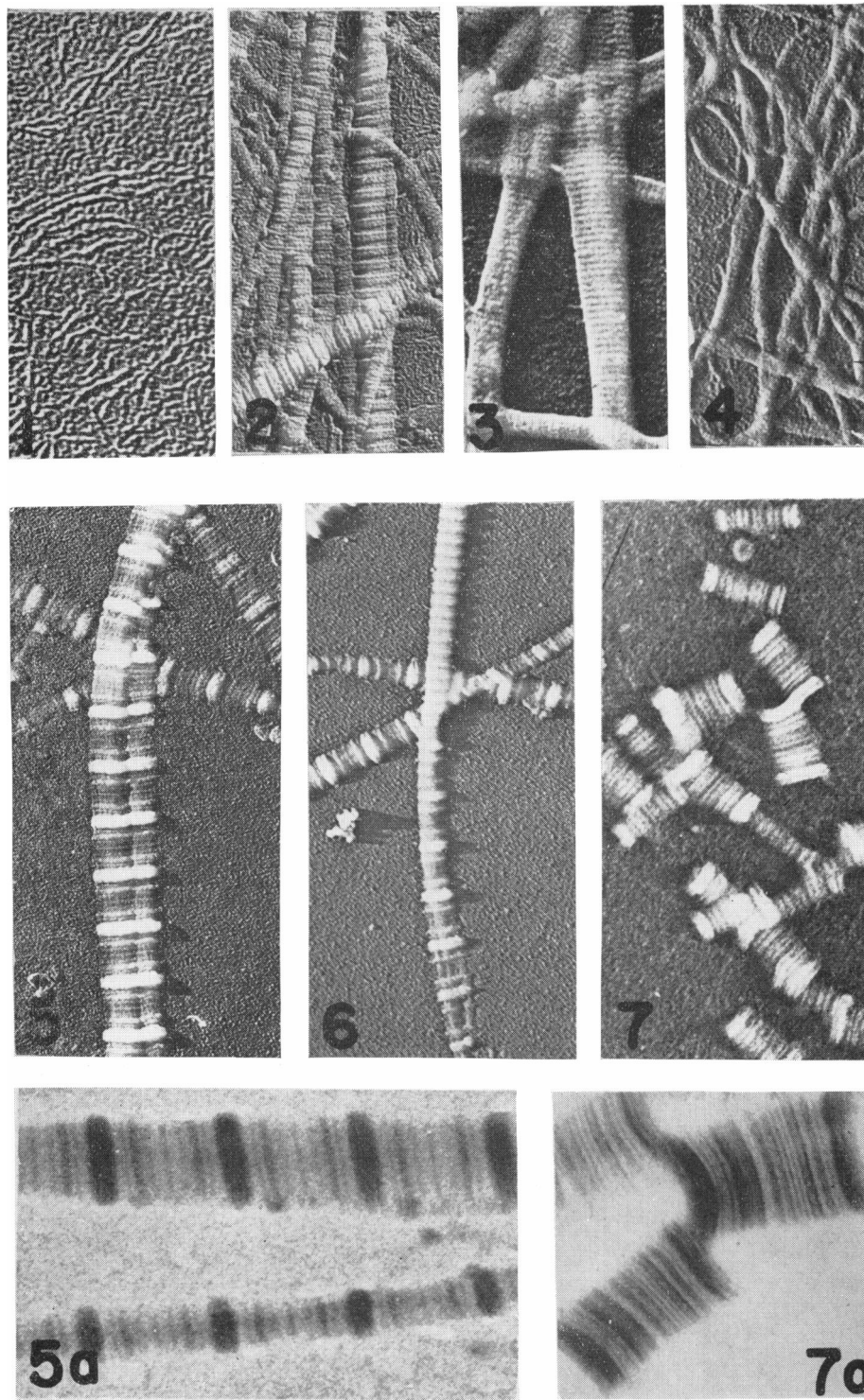


PLATE II [LEGEND ON OPPOSITE PAGE]

micrographs have demonstrated that these structures are usually protoplasmic extensions and cannot be identified as collagen (Plate I). The formation of fibrils close to such protoplasmic processes or close to the cell surface could readily cause confusion on the histological level. It would not be too surprising, however, if under certain special circumstances collagen fibrils were formed inside the cell.

The case for extracellular formation rests on direct observations in tissue culture¹⁸ and in the rabbit ear chamber¹⁹ and by inference, from *in vitro* experiments demonstrating reconstitution of fibrils from collagen solutions in the test tube²⁰⁻²³. More than 50 years ago it was discovered that a portion of certain tissues such as tendon and skin collagen may be dissolved in dilute acid and reconstituted to fibrils simply by the addition of salt or neutralization to physiologic ionic strength and pH. These fibrils, as seen in the electron microscope, show all the detailed fine structure of the native collagen (Plate II, Figures 1 and 2). It is also possible to reconstitute collagen from solution by various manipulations²⁴⁻²⁷ in the form of structures with entirely different morphology (Plate II). Fibrils without period or with axial periodicities of 220 Å or 2500 Å may be precipitated from solution. Sheets of collagen with the native 640 Å period or the artificial 2500 Å may be formed from solution and also small regular crystallinities 2500 Å long with an intricate and reproducible fine structure. These different forms

PLATE II—(From Gross, J. J. *Biophys. Biochem. Cytol.*, 2:261, 1956. Suppl. Reprinted by permission.)

Fig. 1. Acetic acid solution of collagen, pH 5.0; chromium-shadowed. X 50,000.

Fig. 2. Collagen fibrils precipitated from collagen solution by 1 per cent NaCl. Average period 650 Å. X 32,000.

Fig. 3. Collagen fibrils precipitated from collagen solution by 2 per cent NaCl. X 45,000.

Fig. 4. Collagen fibrils precipitated from collagen solution by 3 per cent NaCl. X 30,000.

Fig. 5. FLS (Fibrous Long Spacing) precipitated from collagen solution by addition of serum α_1 acid glycoprotein, 0.06 per cent, followed by dialysis. (Other inducing agents will produce the same structure; only the collagen is specific.) X 30,250.

Fig. 5a. Same but stained with phosphotungstic acid. Note symmetrical fine structure. X 103,000.

Fig. 6. Intermediate fibril type, native collagen period transforming to FLS. Induced by addition of 0.03 per cent glycoprotein. X 26,000.

Fig. 7. SLS (Segment Long Spacing) precipitated from collagen solution by addition of 0.2 per cent ATP. X 38,500.

Fig. 7a. Same as Fig. 7 but stained with phosphotungstic acid. X 117,000.

of collagen represent different states of aggregation of the highly asymmetric collagen molecule, called tropocollagen²⁵⁻²⁶. The inducing agents are relatively non-specific. This fundamental unit of collagen is a rigid rod about 2800 Å in length and 17 Å wide as measured in solution by physical chemical methods²⁸ and visualized directly in the electron microscope²⁹. The structure of the molecule itself is under intensive investigation primarily by the methods of x-ray diffraction and physical chemistry¹⁶.

These studies indicate that a solution of collagen molecules may be aggregated reversibly, without the intervention of cells or enzymes, into a variety of structural forms including that of the native collagen fibril found in tissues. Indeed, this is the form which almost invariably occurs when the pH and ionic strength are in the physiological range. All the other types require additional conditions. The type of fibril with a 220 Å period has been reported in embryonic tissues and in tissue cultures, the structureless form has been seen in cartilage and nerve, and something resembling the "fibrous long spacing" with periods of the order 1800 Å have been seen in cornea³⁰. These unusual forms of collagen may reflect unusual environmental conditions at the time of their formation.

Collagen has generally been considered to be an insoluble substance and has thus been classified among the scleroproteins, however some of its most interesting properties have been discovered in the course of studies on its solubility in aqueous media. The observations on the properties and behavior of some forms of collagen dissolved in dilute organic acids have been discussed. It is the biological implications which will concern us now. It was noted by the early French workers that tendon collagen which was soluble in dilute unbuffered organic acids in young animals became more insoluble with age. Orekhovitch and colleagues^{31, 32} observed that the amounts of collagen extracted from animal skins with citrate buffers at acid pH diminished with age. This collagen fraction which could be readily precipitated as needle-like crystals and fibers they called "procollagen" implying precursor of the tissue fibers. With the exception of certain small differences^{33, 34}, the amino acid composition of "procollagen" is similar to collagen. The Russian workers also observed a more rapid rate of incorporation of C¹⁴ labeled glycine into "procollagen" than into the insoluble collagen, the latter being quite inert metabolically.

In 1951 Highberger, Gross and Schmitt²⁴ reported the extraction of collagen from fresh connective tissue with cold, mildly alkaline phosphate buffer and the reconstitution of this collagen fraction to typical fibrils *in vitro*. Harkness, Marko, Muir and Neuberger³⁵, using essentially the same procedure, subsequently demonstrated that this collagen fraction exhibited a relatively rapid rate of incorporation and "turnover" of C¹⁴ labeled glycine. Its metabolic activity was much higher than that of the collagen fraction extracted by acid citrate buffer, the "procollagen" of Orekhovitch. The remaining insoluble collagen of the skin was comparatively inert in terms of turnover. Gross, Highberger and Schmitt³⁶ then described the extraction of collagen from skin in cold salt solutions at physiological pH and at both high and physiological ionic strength (including serum ultrafiltrate), and showed that the collagen extracted thereby could be precipitated in the form of native fibrils simply by warming to 37°C; almost simultaneously Jackson and Fessler³⁷ reported similar observations. Jackson³⁸ then demonstrated again by isotope incorporation studies, that the neutral salt extractable collagen obtained from the actively growing carrageenin granuloma had a rapid "turnover" rate as compared to the citrate soluble and insoluble collagen fractions. Gross^{39, 40} has since shown that the amount of neutral salt extractable collagen in the skin of guinea pigs is dependent on growth rate and is sharply reduced by several days of weight loss induced by fasting. After one or two weeks on a limited diet, which maintains static body weight, very little neutral salt extractable collagen can be obtained from skin. At least five days of renewed weight on an *ad lib* diet is required for the reappearance of this collagen fraction. The evidence is strong that the neutral salt extractable fraction is a newly synthesized collagen, probably produced less than 48 hours before extraction. Some of it may be in molecular dispersion in the ground substance and some in the form of loosely aggregated fibrils. The collagen isolated from such extracts, and purified, may be readily polymerized to typical fibrils simply by warming the solution in the test tube³⁶ (Plate III). It would appear that most of the extracted collagen molecules are complete and capable of association to form fibrils without further enzymatic modification or the intervention of any other substances, including mucopolysaccharides or glycoproteins—at least *in vitro*. It is of interest that the rate at which collagen fibrils will form *in vitro* by warming is strongly influenced

PLATE III—(From Gross, J. and Kirk, D., J. Biol. Chem.
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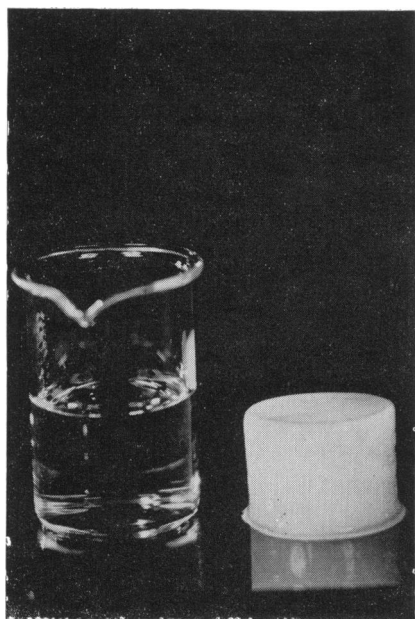


Fig. 1

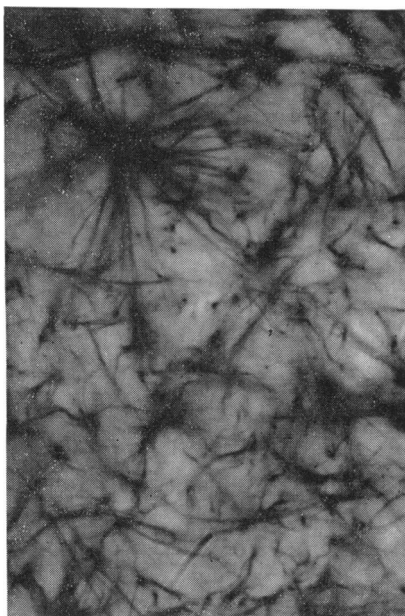


Fig. 2a

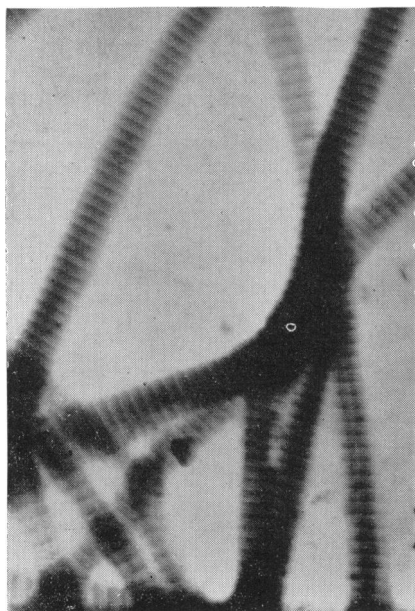


Fig. 2b

Fig. 1. Photograph of beaker of neutral phosphate solution of calf skin collagen and the rigid gel formed therefrom by warming to 37°C for 30 minutes.

Fig. 2a. Histological photomicrograph of gel stained with Mallory's aniline blue connective tissue stain, showing random orientation of fibers.

Fig. 2b. Electron micrograph of gel fragmented and stained with phosphotungstic acid. Magnification 17,200 X.

by the presence of low concentrations of small molecules such as urea, certain amino acids, small organic and inorganic anions and not obviously affected by hyaluronic or chondroitin sulfuric acids^{27, 41}. Whether or not these observations can be extended to the intact tissues is yet to be demonstrated.

What, then, is the relationship between the collagen extracted in dilute acetic acid, in citrate buffer, in neutral salt solutions of different concentrations and the insoluble collagen? Do they each represent a slightly different molecular species in terms of composition or molecular configuration? In the opinion of this writer the small differences in amino acid composition observed may be the result of variable amounts of impurities having to do with the method of preparation. The observations that acid extracted collagen can be readily transferred to a cold neutral medium and remain dissolved—and vice versa—and will show similar behavior with regard to fibril formation, suggest that the molecules are essentially the same. This likelihood is further supported by the similarity of the measured dimensions of the collagen molecule (by physical chemical means) in the several media. There is the possibility, however, that one fraction of the collagen extracted from fresh skin with cold 0.14 *M* NaCl is different. Fessler⁴² has separated the collagen of such extracts into three fractions, one of which, although it has the same molecular dimensions of the other two, will not form fibrils in the usual manner.

Some recent observations by the writer^{43, 44} suggest that the differences between the several extractable collagens relate to the state of aggregation of the collagen molecules in the tissue and this in turn is a function of the time at which they were synthesized and aggregated into extracellular structures. Other factors may also be involved. It was observed that collagen fibrils recently formed from neutral solutions of purified collagen by warming to 37°C would redissolve on cooling. However, the longer they remained at the elevated temperature the more insoluble they became; within 24 hours of incubation, relatively little collagen would redissolve upon cooling. Collagen fibrils so formed at physiological ionic strength became insoluble more rapidly than those formed in hypertonic media. It was also observed that after a two week period of incubation at 37°C of similar preparations, the fibrils were no longer soluble in citrate buffer at pH 3.5 (Orekhovitch's medium).

Extrapolating back to the intact tissues these data suggest that the collagen which dissolved in cold neutral salt solutions at physiologic ionic strength was probably synthesized within several hours of extraction. That fraction removed at higher salt concentrations (ca, 0.45 *M* NaCl) had been synthesized within 1-2 days of extraction and the acid citrate extractable collagen may have been synthesized within 1-2 weeks of extraction. Thus, the major difference between the several soluble collagens would be their age. The mechanism of increasing insolubility may involve several factors, one of which is simply the increasing perfection of fit between collagen molecules as they gradually pack together into a more stable association in striated fibrils. Increasing numbers of secondary crosslinks would form (hydrogen bonds, electrostatic bonds between charged groups, Van der Waals forces) making disruption of the fibril increasingly difficult. The presence of other strongly polar and charged molecules, both small and large, perhaps including other proteins and mucopolysaccharides may aid in this process. However, this phenomenon can be observed to occur in preparations of highly purified collagen.

It seems likely that the diminished extractability of collagen in the aging animal^{32, 45} is mainly a function of cessation of growth. Growth rate experiments described earlier suggest that collagen synthesis, at least in skin, ceases when weight gain ceases. The collagen, newly formed during the earlier growth phase, becomes increasingly insoluble with time by the mechanisms proposed above. One might speculate that if this phenomenon is a general occurrence when rigidly structured, sterically specific molecules are closely associated for prolonged periods of time, it may have broad significance with regard to the aging process.

To summarize, it seems likely that the fibroblast secretes the collagen molecules into the amorphous, extracellular ground substance and there, under the influence of the physiologic ionic atmosphere, temperature and perhaps certain rate regulating factors, the molecules aggregate spontaneously to form fibrils. There may, of course, be several earlier precursor forms, preceding the finished molecule, which have not yet been observed. The role, if any, played in this process by the non-collagenous components of the ground substance, such as mucopolysaccharides and other proteins, is still obscure. It is possible that these substances are important in the geographic ordering of the fibrils into bundles on the histological level or perhaps play some part

in regulating the rate of fibril formation and perhaps fibril size in the ground substance; but these are frank speculations at this time.

WOUND HEALING AND CONTRACTION

One of the oldest and still vigorously investigated problems in surgery is the mechanism of wound healing and the associated process of contraction. The early gross histological and tensile strength studies of healing wounds and the relationship of age, nutrition, species, etc. form a massive bibliography. In very recent years the broad applications of chemical methods have led to analyses of the sequence of chemical changes in healing and contracting wounds⁴⁶⁻⁴⁸. Most of this work has been based on the intuitively derived and generally accepted concept that changes in the central granulation tissues of an open wound result in a pulling together of the wound edges. The mechanism has been variously ascribed to diminution in total wound content, contraction of collagen, dehydration, and more recently by dynamic contraction resulting from contractile activity of the fibroblasts in the granulations.

In our laboratory the process of contraction in open skin wounds in guinea pigs has been reinvestigated by Grillo, Watts and Gross⁴⁹⁻⁵¹ both chemically and morphologically.

Full thickness excisions (including panniculus carnosus) were made on each flank of groups of guinea pigs and the rate of contraction in area and the linear movement of the edges measured daily. These were correlated with the total wet and dry weight of the granulation tissue within the wound edges, the water content, collagen (hydroxyproline), hexosamine, and non-collagenous protein (measured as tyrosine). Our daily measurements, in contrast to the earlier data⁴⁶⁻⁴⁷, indicated a sharp and considerable rise in total wound tissue, wet and dry, and in collagen content between day 4 and 6, followed by as rapid a fall between days 6-7 and 10. This unexpected up and down fluctuation in wound content occurred during the period of maximum rate of contraction. Thus, there seemed to be no direct correlation between contraction and wound contents. This significant fluctuation was not observed in earlier studies probably because of the longer time intervals between observations.

This observation raised doubt concerning the direct role of the central granulations in bringing about approximation of the wound

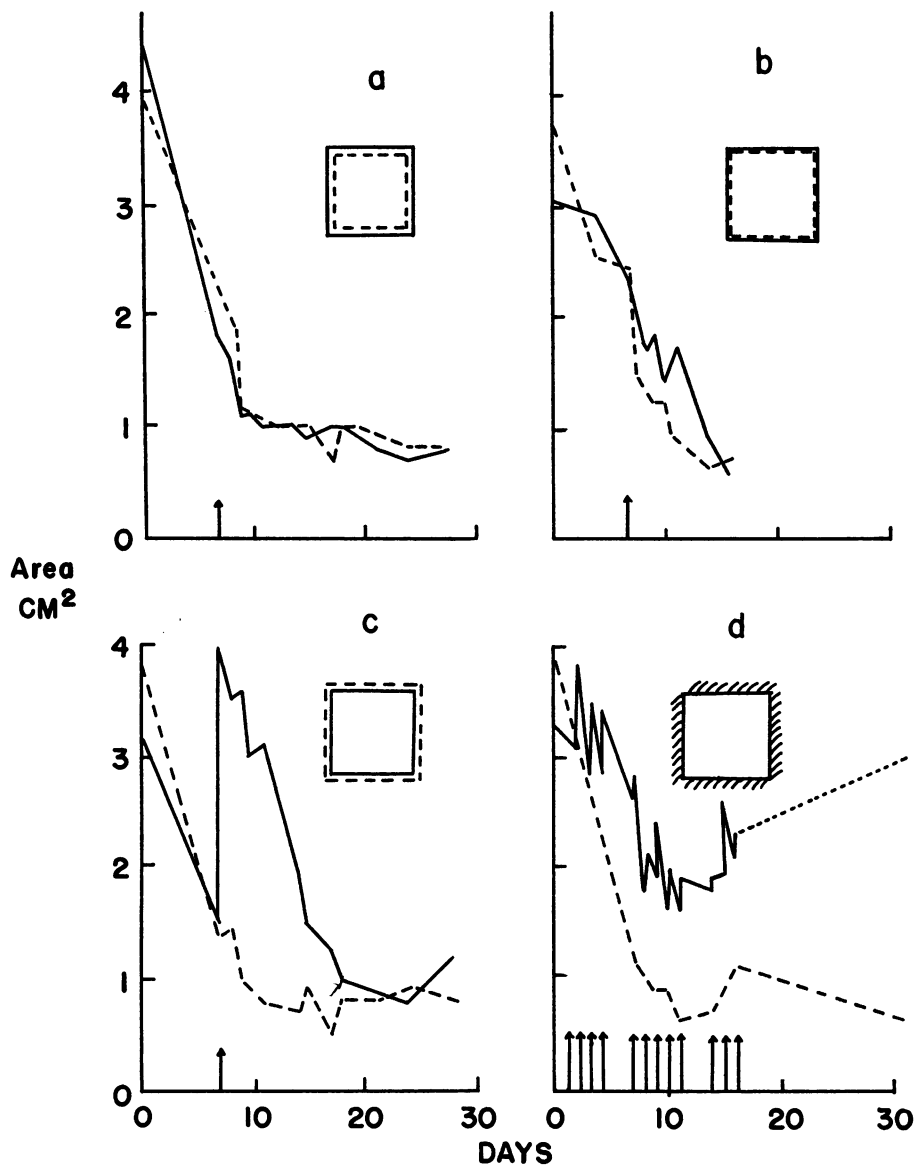


PLATE IV—Plot of change in area of excised square wound in guinea pig skin as a function of time in days. Dotted lines in graph represent contraction in control wound; solid lines represent course of contraction in manipulated wound. Solid line of square represents edge of original wound and dotted line of square represents edge of experimental excision after seven days of healing. Arrows indicate times of manipulation.

- (a) Granulations excised to within a mm. of advancing wound edge.
- (b) Granulations excised to advancing wound edge.
- (c) Granulations plus one mm. of advancing wound edge excised.
- (d) Repeated raising of edge of advancing wound margin.

edges. In a series of gross morphologic experiments the central granulations were completely excised within less than a millimeter of the wound edges during the period of maximum contraction rate. Excision was repeated as soon as new growth appeared. No change in either rate or extent of contraction of the wound was observed nor was there any distraction of the wound following excision as compared with control wounds in the same animal (Plate IV-a). Excision of granulations up to the advancing skin edges produced no interference in contraction rate (Plate IV-b). When one millimeter of the wound edge plus the granulations were removed, immediate large distraction of the edges occurred returning the wound to a size larger than the original area (Plate IV-c). Contraction then proceeded again at about the same rate as that of the initial wound. Removing the edge but leaving the central granulations intact had the same effect. In order to determine whether or not a sphincter mechanism in the wound edge was responsible, the edges of actively contracting wounds were separated from the central granulations then raised by sharp dissection from the base (Plate IV-d). Immediate increase in wound area occurred. If this was repeated on successive days, distraction occurred each time and the final area of the wound after 30 days was about the same or larger than that at the time of the first intervention. The inescapable conclusion was that the central mass of granulation tissue within the edges of the advancing skin was not necessary for contraction of an open skin wound. The machinery for contraction must reside in a very narrow "picture frame" of new tissue underlying the margin of skin at the edge of the wound, perhaps projecting slightly into the granulations. Histological and other studies are now in progress on the "picture frame". Our current hypothesis for the mechanism of contraction places the responsibility more on the cells in the wound margin rather than on some process involving the extracellular substances directly.

In summary, we have large masses of descriptive information concerning the surface appearance and behavior of diseases thought to involve connective tissue, but very little knowledge which leads to an understanding of mechanisms. One hopes that a better understanding of the fundamental nature and behavior of connective tissues, their composition, structure, synthesis, degradation and function may lead to fruitful directions of study into the pathogenesis of diseases affecting them.

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